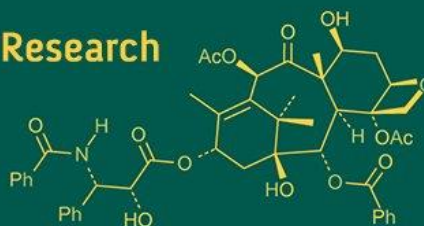


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2026): 5.29
IJABR 2026; SP-10(6): 675-681
www.biochemjournal.com
Received: 09-04-2026
Accepted: 13-05-2026

Rishee K Kalaria
ASPEE Shakilam
Biotechnology Institute,
Navsari Agricultural
University, Surat, Gujarat,
India

Hiren V Patel
ASPEE Shakilam
Biotechnology Institute,
Navsari Agricultural
University, Surat, Gujarat,
India

Nirav V Radadiya
Main Sorghum Research
Station, Navsari Agricultural
University, Surat, Gujarat,
India

Bharat K Davda
Main Sorghum Research
Station, Navsari Agricultural
University, Surat, Gujarat,
India

Tushar V Ghevariya
Main Sorghum Research
Station, Navsari Agricultural
University, Surat, Gujarat,
India

Hiren N Der
Main Sorghum Research
Station, Navsari Agricultural
University, Surat, Gujarat,
India

Corresponding Author:
Rishee K Kalaria
ASPEE Shakilam
Biotechnology Institute,
Navsari Agricultural
University, Surat, Gujarat,
India

Molecular characterization of fall armyworm, *Spodoptera frugiperda* (J. E. Smith) using mitochondrial COI gene infesting sorghum in Gujarat region

Rishee K Kalaria, Hiren V Patel, Nirav V Radadiya, Bharat K Davda, Tushar V Ghevariya and Hiren N Der

DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i6Sh.8690>

Abstract

The invasive and destructive fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) has spread to almost every Indian state and seriously damages sorghum crops all over the world. The genetic diversity of *S. frugiperda* populations infesting sorghum in four agro climatic areas of Gujarat were evaluated in this study. While phylogenetic clustering showed genetic homogeneity and substantial gene flow across regions, molecular analysis of COX I genes proved species identity with negligible intra-specific divergence. Two distinct populations of the C-strain of *S. frugiperda* were found to be prevalent in sorghum in the Gujarat region, according to subsequent phylogenetic research. Both the clades exhibited close similarity with geographically isolated locations indicating possible source of introduction as 2 haplotypes were prevalent in fields of sorghum from Gujarat. Eight sequences and four barcodes were added to GenBank and BOLD respectively, enhancing international reference databases. Further research is needed to confirm these tentatively identified strains of *S. frugiperda*, which would be helpful for host-plant identification, proper monitoring, and effective control of such pests.

Keywords: *Spodoptera frugiperda*, sorghum, integrated pest management, barcoding, phylogeny and COX I gene

Introduction

Often referred to as "jowar" or "milo," sorghum, *Sorghum bicolor*, is a highly drought-tolerant, adaptable cereal grain and staple food. Sorghum is a major crop in Asian and African nations like Nigeria and India, where it is used to create bread and porridges. In many underdeveloped and semiarid areas, sorghum is the main source of human nutrition and energy (Rooney and Waniska, 2000) [15]. About 52.80 million tonnes of sorghum were produced worldwide in 2023-2024. With 4.40 million tonnes of sorghum planted on 3.97 million hectares in 2023-2024, India ranks fourth in the world for overall sorghum production. The majority of the sorghum was produced during the *rabi* season. The Government of India's third advance estimate for 2023-2024 states that the sorghum crop will yield 47.42 lakh tonnes, up from 38.14 lakh tonnes in 2022-2023 (Anon., 2023) [1]. In various Indian agroecosystems, it is home to around 150 insect species. According to Nwanze *et al.* (1995), the sorghum midge, shoot fly, and stem borer have become important pests in India and are responsible for around 32 percent of production loss. The autumn armyworm, *S. frugiperda* (J. E. Smith), is a newly introduced pest that poses a serious danger to global cereal production and is reducing sorghum yield by 70 percent (Venkateswarlu *et al.*, 2018; Harrison *et al.*, 2019) [17, 5].

Spodoptera frugiperda (J.E. Smith) (Lepidoptera: Noctuidae), the tropical American bug known as the fall armyworm (FAW), is an invasive pest that results in large financial losses. This harmful pest might seriously injure about 100 plant species from 27 families, including the maize crop and other graminaceous crops like millet, sorghum, rice, wheat and sugarcane (Goergen *et al.*, 2016) [3]. Sorghum, the second most affected crop after maize, is severely harmed by fall army worms, which are polyphagous and highly destructive parasites.

It has been determined that yield losses from twelve maize producing countries range from 8.30 to 20.60 million tons per year. This pest has been found on maize crops in several areas of the states of Telangana and Andhra Pradesh since July 2018. In October of 2018, fall armyworm was observed on sorghum and bajra. In the fields of the agricultural research station in Ananthapuramu, 30 percent of the bajra and 70 percent of the sorghum were damaged (Venkateswarlu *et al.*, 2018; Harrison *et al.*, 2019)^[17, 5].

This species is well-known for its wide host range, fast rate of reproduction, tolerance to traditional pesticides and adaptability to a variety of circumstances. Depending on the host plant's desire, FAW is composed of two strains: the rice strain and the corn strain. These strains differ genetically, with a large proportion of genomic divergence, yet having comparable morphologies. With two dominant strains, "C" and "R", the FAW has developed into a super pest. While the rice strain "R" primarily targets rice and other grasses, the corn strain "C" affects maize, sorghum and cotton. (Venkateswarlu *et al.*, 2018; Harrison *et al.*, 2019; Dash *et al.*, 2024)^[17, 5, 4].

Because of the intraspecific and interspecific variation among FAW strains, precise strain identification is essential in field studies of FAW populations. The two strains of FAW are mainly distinguished by polymorphisms in the mitochondrial gene cytochrome c oxidase (Nagoshi, 2010)^[10]. Because most invaded areas have few haplotypes and limited genetic diversity, which may indicate a recent introduction from a common source of the FAW population, monitoring of the crops at risk in these areas may be impacted (Nagoshi *et al.*, 2018 & Nagoshi *et al.*, 2020)^[11, 12]. Molecular genetic markers are one of the most reliable methods to differentiate between the many strains of FAW, even though their morphometric traits can be evaluated based on the shapes of their wings. As a result, DNA-based methods provide a dependable and all-purpose identification tool that may be applied throughout life. As a result, DNA barcoding is increasingly being recommended and used globally to identify invasive species. To identify species that are important from an ecological and economic perspective, a portion of the mitochondrial cytochrome c oxidase subunit 1 (COI) gene is used during the barcoding process. (Venkateswarlu *et al.*, 2018; Harrison *et al.*, 2019; Ramos-Hernández *et al.*, 2023)^[17, 5, 14].

FAW is a global concern because it could jeopardize the lives and food security of many small-scale farmers in Asia and India. The purpose of this study was to increase research on the genetic diversity of FAW populations in south Gujarat. The current work uses COX I gene sequences to describe the genetic diversity of *S. frugiperda* populations obtained from four different sorghum growing areas spanning different agroclimatic zones of Gujarat. It is anticipated that these discoveries will aid in the creation of sustainable management plans, population monitoring, and more precise pest identification.

Materials and Methods

Sample Collection and Preservation

S. frugiperda larvae were gathered as specimens from four sorghum-growing sites spread across different Gujarat agroclimatic zones during the trial periods of 2024-2025. At the height of the infestation in the *kharif* of 2024-2025, the following sites were chosen for the study at Main Sorghum Research Station (MSRS), NAU, Surat and one farmer field

each from Surat, Tapi and Junagadh district. For molecular analysis, representative specimens were preserved in 70% ethanol and stored at -20 °C until DNA extraction. (Babu *et al.*, 2019; Ramos-Hernández *et al.*, 2023)^[2, 14].

Genomic DNA Isolation

A modified CTAB approach was used to extract the genomic DNA of *S. frugiperda* obtained from four separate locations. Proteinase K and RNase treatments were followed by chloroform: isoamyl alcohol purification and isopropanol precipitation. DNA purity (A260/A280) was evaluated using NanoDrop spectrophotometry, and DNA integrity was verified by 0.8 percent agarose gel electrophoresis. Samples were kept for further molecular analysis at -20 °C. (Ramos-Hernández *et al.*, 2023; Lebody *et al.*, 2024; Kalaria *et al.*, 2025)^[14, 8, 6].

PCR amplification and purification

Universal primers were used to amplify mitochondrial COX I areas in accordance with procedures by Nagoshi *et al.* (2018)^[11], Babu *et al.* (2019)^[2] and Kalaria *et al.* (2025)^[6]. The master mix, primers, DNA template, and nuclease-free water were all included in each 25 µl PCR reaction. The cycling program consisted of initial denaturation at 94 °C for 1 min, 30 cycles of 94 °C for 35 s, 58 °C for 50 s, and 72 °C for 50 s, followed by a final extension at 72 °C for 5 min. Ethidium bromide-stained 2 percent agarose gels were used to confirm amplicons. The Sanger technique was used to sequence the PCR products after they were purified using ethanol-sodium acetate precipitation and resuspended in Hi-DiTM formamide. After being trimmed and aligned, high-quality reads were utilized for molecular analysis. (Sathyan, *et al.*, 2022; Mohapatra *et al.*, 2023; Ramos-Hernández *et al.*, 2023; Kalaria *et al.*, 2025)^[16, 9, 14, 6].

DNA Sequence assembly and phylogenetic tree analysis

COX I readings were cleaned in BioEdit v7, processed in MEGA 12, and put together into consensus sequences using Sequencher v5.3. Accession numbers were allocated to validated sequences that were submitted to NCBI GenBank, and identities were verified using BLASTn (≥98% similarity) (Mohapatra *et al.*, 2023; Kalaria *et al.*, 2025)^[9, 6]. ClustalW alignments were used to examine barcode gap and genetic divergence. Maximum Likelihood was used to infer phylogenetic relationships (MEGA 12, 1000 bootstraps) (Sathyan, *et al.*, 2022; Mohapatra *et al.*, 2023; Ramos-Hernández *et al.*, 2023; Kalaria *et al.*, 2025)^[16, 9, 14, 6].

Submission to the Barcode of Life Data System (BOLD)

The Barcode of Life Data System received COX I sequences, metadata, photos, and trace files in order to facilitate international data exchange and precise species identification by DNA barcoding analysis (Kalaria *et al.*, 2025)^[6].

Results and Discussion

Specimen collection and Molecular identification of *S. frugiperda* Using COX I Genes

To gather *S. frugiperda* larvae, field surveys were carried out in four main sorghum-growing localities in Gujarat: MSRS, NAU, Surat and one farmer field each from Surat, Tapi and Junagadh district. Mature, fully developed larvae were gathered at random from several fields, preserved in 70 percent alcohol, and their geographic location longitude and

latitude was noted. The Division of Plant Biotechnology, ASBI, NAU, Surat conducted molecular research to examine *S. frugiperda* intra-species genetic diversity of specimen collected across several agroclimatic zones (Fig 1, Fig 2a, Fig 2b, Fig 2c, Fig 2d).

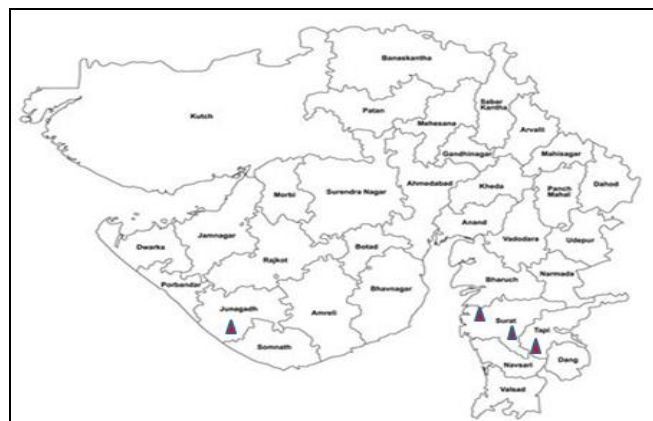


Fig 1: Different sampling sites explored for *Spodoptera frugiperda* isolate from sorghum collection in Gujarat



Fig 2 a: Larvae collected randomly from Mangrol field of Surat district



Fig 2 b: Larvae collected randomly from Main Sorghum Research Station, NAU, Surat



Fig 2 c: Larvae collected randomly from farmer field Junagadh district



Fig 2 d: Larvae collected randomly from farmer field (Songadh) Tapi district

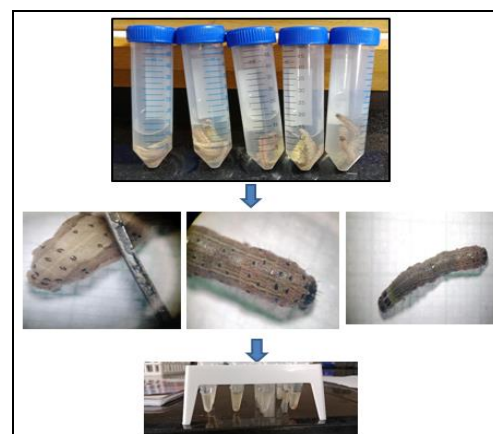


Fig 3: Morphological identification and DNA isolation from larvae collected from various field

Similarly, *S. frugiperda* specimens were collected in corn fields (INIFAP-CEHUI) during 2021-2022 (Ramos-Hernández *et al.*, 2023) ^[14]. Maize fields infested by fall armyworm were surveyed and fall armyworm larvae were collected from maize growing area of middle Gujarat at peak infestation level in *kharif* 2021 and *rabi* 2021-22 (Mohapatra *et al.*, 2023) ^[9].

Genomic DNA Extraction and PCR amplification

For molecular analysis, specimens were kept at -20 °C and maintained in 70 percent ethanol (Fig 3). A modified CTAB procedure (Fig 4) optimized for yield and integrity was employed to extract genomic DNA from pooled samples. NanoDrop readings ($A_{260}/A_{280} = 1.79-1.85$) demonstrated good purity, appropriate for PCR amplification, DNA barcoding, and sequencing, and Agarose gel electrophoresis verified intact, high-molecular-weight DNA (Kalaria *et al.*, 2025) ^[6].

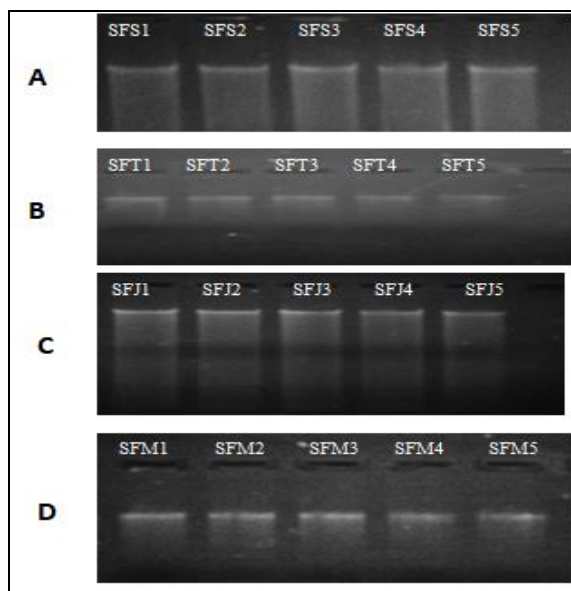


Fig 4: DNA isolation from Larvae collection from A) Surat farm B) songadh (Tapi district) C) Junagadh district D) Mangrol (Surat district)

Using primers *COI101F/COI911R*, mitochondrial *COXI* (~700 bp) regions from *S. frugiperda* obtained from four locations in Gujarat were successfully amplified (Nagoshi *et al.*, 2018; Babu *et al.*, 2019) [11, 2]. In line with *S. frugiperda* investigations, clear, distinct bands on 2 percent agarose gels (Fig 5) validated primer specificity and PCR efficiency

(Nagoshi *et al.*, 2020) [12]. All loci produced high-quality bidirectional reads when sequenced using gene-specific primers and the BDT v3.1 Cycle Sequencing Kit on an ABI 3730xl Genetic Analyzer, offering a solid foundation for phylogenetic and genetic diversity analysis.

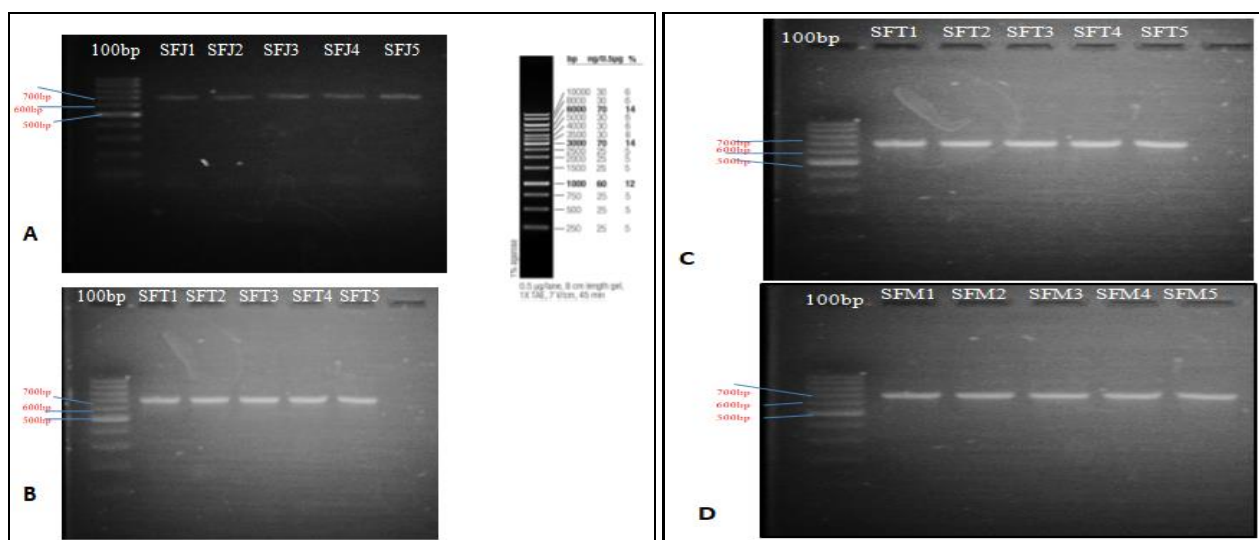


Fig 5: Amplification of partial *COI* gene (~700bp) from Larvae collection from A)Surat farm B)songadh (Tapi district) C)Junagadh district D) Mangrol (Surat district)

Ramos-Hernández *et al.* (2023) [14] and Kalaria *et al.* (2025) [6] also reported a single, thick and transparent band with amplicons of roughly 700 bp is produced by molecular characterisation of the barcoding *COI* region of the *S. frugiperda* discovered in Tabasco. Similarly Mohapatra *et al.* (2023) [9] and Lebody *et al.* (2024) [8] also reported PCR amplification resulted in a ~700 bp fragment among 33 FAW strains utilizing cytochrome oxidase (*COX1*) sequences.

DNA Sequence Assembly

BLASTn-verified consensus sequences constructed in Sequencher v5.3 demonstrated 100-99.84 percent similarity to *COX I S. frugiperda*. BioEdit v7 quality tests verified intact ORFs in accordance with morphological

identification. According to earlier research, the validated gene regions particularly *COX I* proved useful for species identification (Mohapatra *et al.*, 2023; Lebody *et al.*, 2024; Kalaria *et al.*, 2025) [9, 8, 6]. All sequences were submitted to GenBank using the following accession numbers: JPV156122.1, PV156132.1, PV156480.1, PV156481.1, PV156447.1, PV156448.1, PV156493.1, and PV156519.1.

Phylogenetic Analysis and Genetic Distance

Using Maximum Likelihood in MEGA12, phylogenetic relationships between *S. frugiperda* populations from different Gujarat agro climatic zones were deduced from *COX I* sequences (Kumar *et al.*, 2024) [7]. Under the Kimura 2-parameter model, validated by Neighbor-Joining with 1000 bootstraps (Fig 6).

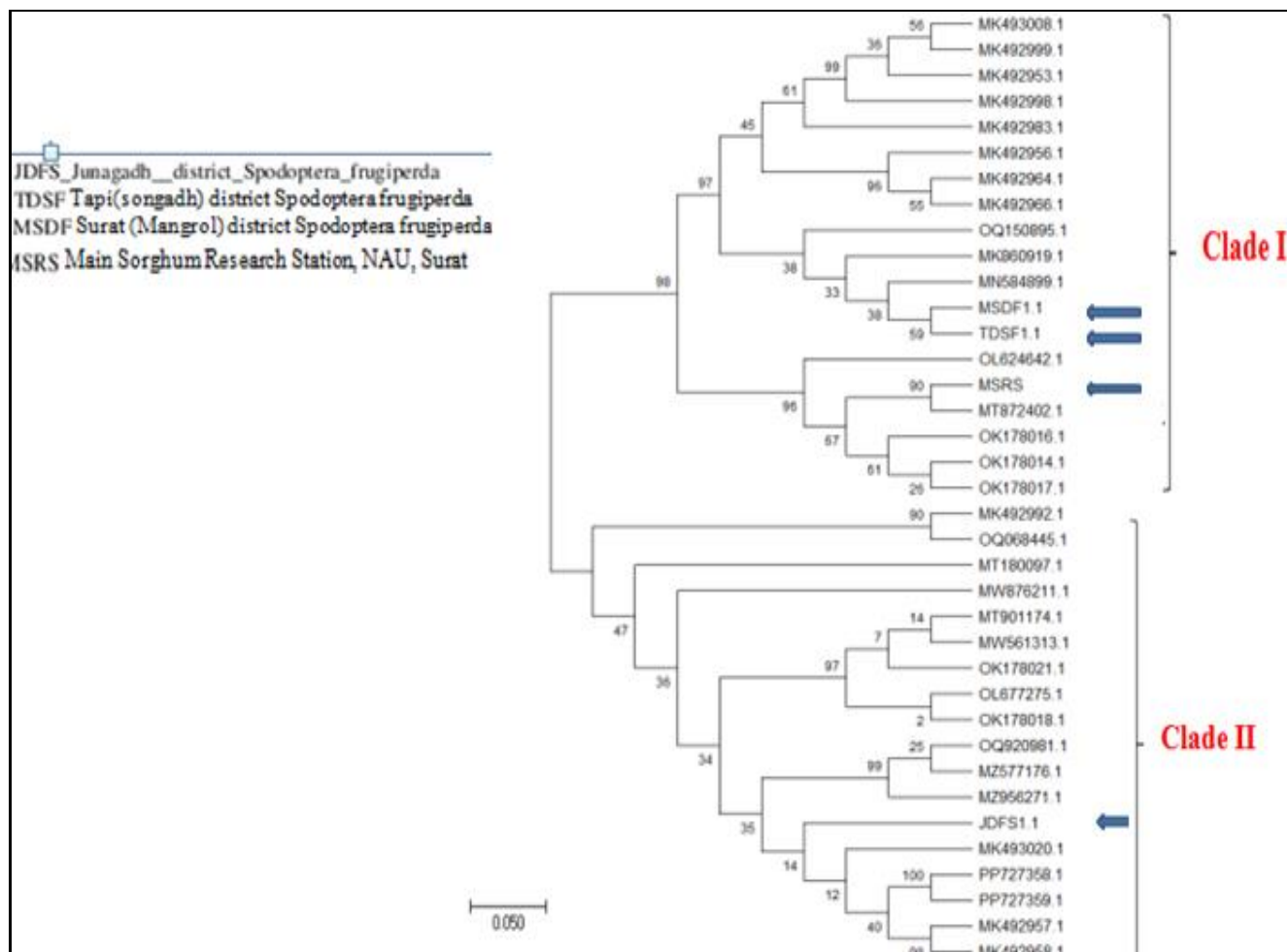


Fig 6: Phylogenetic tree using all *Spodoptera frugiperda* isolate cytochrome c oxidase subunit I (COX1)

COX I created two clades: Clade I included the majority location of Gujarat with little variation (MSRS, NAU, Surat and one farmer field each from Surat, Tapi and Junagadh district), while Clade II included references that were different (Junagadh district), indicating that the two groups had different or ancestral origins. In Junagadh, there was some minor sub-structuring (COX I). The consensus sequences of *S. frugiperda* population from MSRS, NAU, Surat and one farmer field each from Surat, Tapi and Junagadh district exhibited distribution under clade I along with NCBI database isolates viz., MK493008.1, MK492999.1, MK492953.1, MK492998.1, MK492956.1, MK492964.1, MK492966, OQ150895.1, MK860919.1, MN584899.1, OL624642.1, OK178016.1, OK178014.1 and OK178017.1 which majority belong to C strain of Maize while Clade II include consensus sequences of *S. frugiperda* population from Junagadh district along with OQ068445.1, MK492992.1, MT180097.1, MW876211.1, MT901174.1, MW561313.1, OK178021, OL677275, OK178018.1, OQ920981.1, MZ577176.1, MZ956271.1, MK493020.1, PP727358.1, PP727359.1, MK492957.1 and MK492958.1 respectively. Even though the specimens were gathered from districts that were closer to one another, the phylogenetic similarity of the sequences showed that they were more comparable to populations of *S. frugiperda* C-strain that were farther apart. Subsequent phylogeny analysis indicated prevalence of two divergent population of C-strain of *S. frugiperda* in sorghum within Gujarat region. The identification of host-specific strains of *S. frugiperda* may also be aided by single nucleotide polymorphisms (SNPs) in

the genetic marker, which may offer more precise identification information.

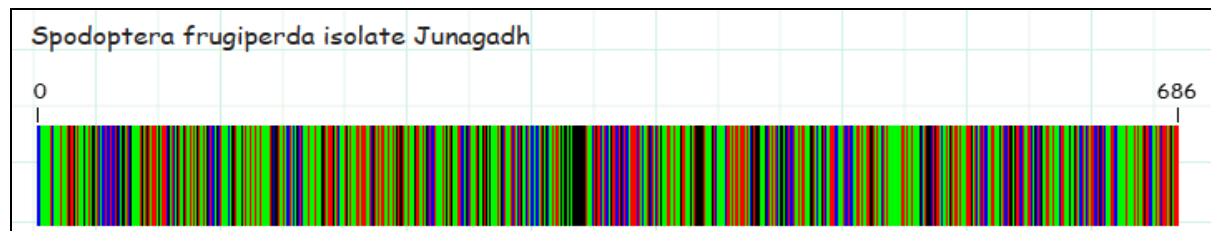
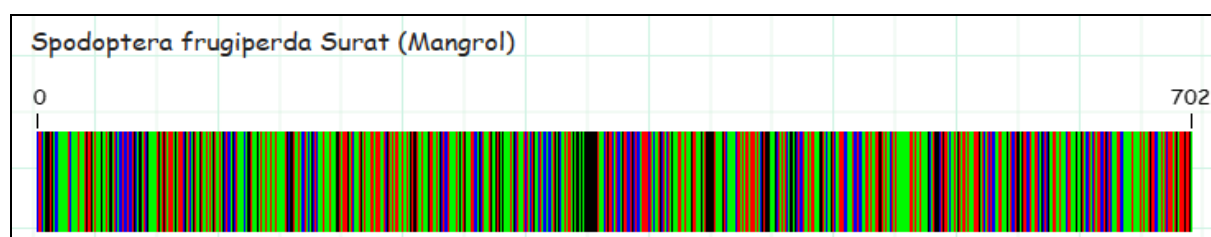
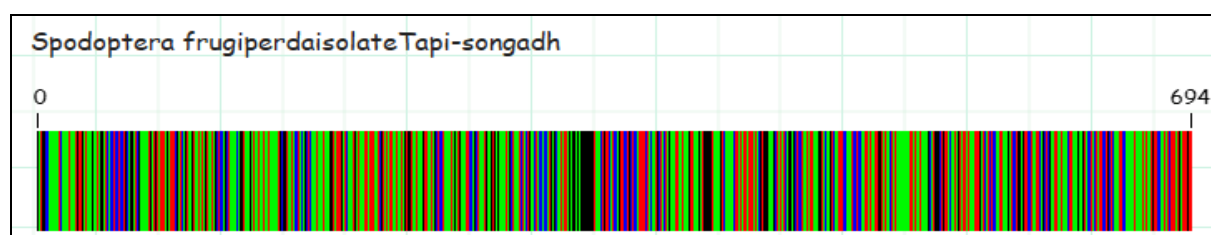
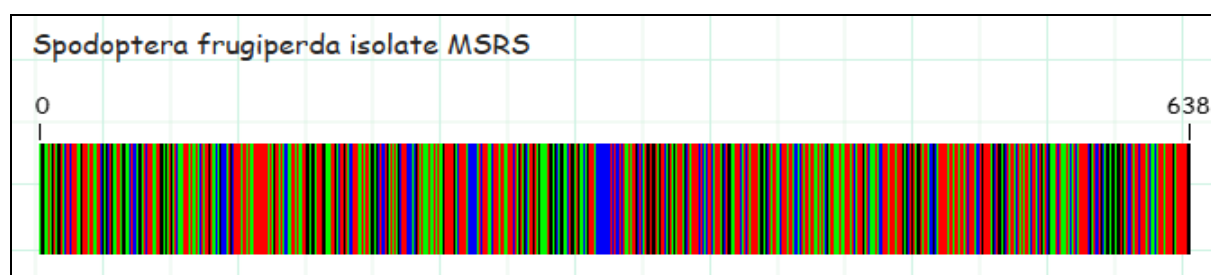
In phylogenetic comparison of the *COI* region, total six sequences used in this study are grouped into the *S. frugiperda* RS clade. An earlier study used two segments of the mitochondrial *COI* gene to confirm that populations from four FAW colonies in Mexico (Durango, Sinaloa, Tamaulipas and Chiapas) belong to the *S. frugiperda* CS clade (Ramos-Hernández *et al.*, 2023) [14]. Mohapatra *et al.* (2023) [9] performed phylogeny analysis used consensus sequences from *S. frugiperda* population characterized under different strains in maize. Similarly, phylogenetic analysis reported as 'C' strain of FAW collected from maize, sweet corn and sorghum crops in India (Sathyan, *et al.*, 2022) [16]. Phylogenetic analysis suggests that the primary sources of *Spodoptera frugiperda* in Egypt likely stem from both Asian and African origins (Lebody *et al.*, 2024) [8].

DNA Barcode Submission to BOLD

The Barcode of Life Data System (BOLD) received specimen-level metadata, including as taxonomy, sequences, trace files, and photos of *S. frugiperda* from four Gujarat regions. For accurate identification and comparison analysis, COX I barcodes were created and unique process IDs were assigned (Table 1, Fig 7). This contribution improves global accessibility and promotes future research on genetic diversity, biogeography, and pest diagnostics, in line with previous barcoding efforts in *Spodoptera litura* (Kalaria *et al.*, 2025) [6].

Table 1: List of accession numbers and process ID generated for the sequences deposited in NCBI and BOLD respectively

Sl. no.	Sample ID	Accession number (NCBI)	Process ID (BOLD)
1	JDFS1	PV156122.1 & PV156132.1	SDP1223001-25
2	TDSFS1	PV156480.1 & PV156481.1	SDP1223002-25
3	MSDFS1	PV156447.1 & PV156448.1	SDP1223003-25
4	SDSFS1	PV156493.1 & PV156519.1	SDP1223004-25

Spodoptera frugiperda* isolate Junagadh district cytochrome c oxidase subunit I (COX1) gene, partial cds**Spodoptera frugiperda* isolate Surat (Mangrol) district cytochrome c oxidase subunit I (COX1) gene, partial cds*****Spodoptera frugiperda* isolate Tapi (songadh) district cytochrome c oxidase subunit I (COX1) gene, partial cds*****Spodoptera frugiperda* isolate MSRS, Surat cytochrome c oxidase subunit I (COX1) gene, partial cds****Fig 7:** COI barcode sequences using *Spodoptera frugiperda* submitted to BOLD database**Conclusion**

Spodoptera frugiperda populations from several locations in Gujarat's sorghum-growing regions were characterized using molecular techniques. Low mitochondrial divergence was revealed by barcode gap analysis, with individuals from four locations closely grouping and just slight divergence seen at the Junagadh area. Lower variability was indicated by pairwise distances that ranged below 1.0 as intraspecific distances between various locations. DNA barcodes for *S. frugiperda* were created using molecular data, and the resulting COI barcode sequences were uploaded to the Barcode of Life Data System (BOLD) together with

pertinent metadata like as taxonomy, specimen photos, sequence information, and trace files. Eight sequences and four barcodes were added to GenBank and BOLD, respectively, enhancing international reference databases. Both the clades exhibited close similarity with geographically isolated locations indicating possible source of introduction as 2 haplotypes were prevalent in fields of sorghum from Gujarat. Therefore, it is essential to develop pest control strategies and detection procedures to reduce the impact of the infestation. Further studies on migratory behavior utilizing integrated mitochondrial markers should be conducted to estimate the source areas or migration

timeframes. These studies have potential for regional and national *S. frugiperda* pest management strategies and are useful for evaluating genetic diversity.

Conflict of Interest

The authors declare that they have no conflict of interest.

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